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Citation for published version:

Hay, J, Carter, D, Lieber, A & Astier, AL 2015, 'Recombinant Ad35 adenoviral proteins as potent modulators of human T cell activation', *Immunology*, vol. 144, no. 3, pp. 453-460. <https://doi.org/10.1111/imm.12391>

Digital Object Identifier (DOI):

[10.1111/imm.12391](https://doi.org/10.1111/imm.12391)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Immunology

Publisher Rights Statement:

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Received Date : 10-Jun-2014

Revised Date : 03-Sep-2014

Accepted Date : 16-Sep-2014

Article type : Original Article

Recombinant Ad35 adenoviral proteins as potent modulators of human T cell activation

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Short title: recombinant adenoviral proteins modulate T cell activation

Keywords: CD46; human T cells; antagonist; agonist; activation; adenovirus 35.

Abbreviations; SCR: short-consensus repeat; Tr1: type 1 regulatory T cells

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/imm.12391

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Summary

The protein CD46 protects cells from complement attack by regulating cleavage of C3b and C3d. CD46 also regulates the adaptive immune response by controlling T cell activation and differentiation. Co-engagement of the T cell receptor and CD46 notably drives T cell differentiation by switching production of IFN γ to secretion of anti-inflammatory IL-10. This regulatory pathway is altered in several chronic inflammatory diseases highlighting its key role for immune homeostasis. The manipulation of the CD46 pathway may therefore provide a powerful means to regulate immune responses. Herein, we investigated the effect of recombinant proteins derived from the fiber knob of the adenovirus serotype 35 (Ad35) that uses CD46 as its entry receptor, on human T cell activation. We compared the effects of Ad35K++, engineered to exhibit enhanced affinity to CD46, and of Ad35K-, mutated in the binding site for CD46. Ad35K++ profoundly affects T cell activation by decreasing the levels of CD46 at the surface of primary T cells, and impairing T cell co-activation, shown by decreased CD25 expression, reduced proliferation and lower secretion of IL-10 and IFN γ . In contrast, Ad35K- acts a potent coactivator of T cells, enhancing T cell proliferation and cytokine production. These data show that recombinant Ad35 proteins are potent modulators of human T cell activation, and support their further development as potential drugs targeting T cell responses.

Introduction

The complement regulator CD46 is a type I transmembrane protein that protects cells from autolysis by its regulation of the activating complement components C3b and C4b [1-3]. The CD46 ectodomain consists of four short-consensus repeat domains (SCR1-4), which are followed by a region rich in serine-threonine-proline (STP region), a transmembrane domain and one of two short cytoplasmic tails produced by alternative splicing [4, 5]. Besides binding to complement components, CD46 also acts as a cellular receptor for many pathogens, and has been dubbed a 'pathogen's magnet' [6]. Pathogens using CD46 as their receptor include several serotypes of adenovirus such as Ad35 [7], the vaccinal strain of measles virus, herpes virus 6 and some strains of bacteria such as *Neisseria gonorrhoeae* and *Neisseria meningitides*, as well as group A *Streptococcus*. Moreover, CD46 is key in the regulation of the adaptive immune response by controlling T cell activation, differentiation and polarity [8-12]. Coligation of CD46 and the TCR provides a strong costimulatory signal to T cells [8, 11], and expression of either the CD46-Cyt1 or the Cyt2 isoform in transgenic mice exerted antagonist effects *in vivo* on T cell-mediated inflammation, which was correlated with different effects *in vitro* on cytokine production and T cell proliferation [10]. CD46 expression at the surface of human T cells is tightly regulated, being shed by matrix metalloproteases upon ligation, and CD46 cleavage is important for its functions [13, 14]. In the presence of IL-2, CD46 drives differentiation of human CD4⁺ T cells towards a regulatory T cell type I (Tr1), characterized by increased production of IL-10 and reduced secretion of IFN γ [9, 15]. This regulatory pathway is altered in T cells from patients with multiple sclerosis, rheumatoid arthritis and asthma, as IL-10 production upon CD46 costimulation is impaired [15-20]. The dysregulation of this pathway in several chronic inflammatory diseases highlights its key role in regulating the homeostasis of the immune response, and therefore makes it an attractive pathway to modulate in these pathologies.

Moreover, most cancer cells upregulate CD46 to avoid complement lysis and inhibition of CD46 on cancer cells may be a promising strategy to enhance antibody-dependent cytotoxicity of therapeutic monoclonal antibodies [21-24].

Ad35K++ is a small recombinant protein derived from the fiber knob of the adenovirus serotype 35 that has been modified to bind to CD46 with picomolar affinity [25]. Previous studies have shown that this CD46 antagonist could transiently remove CD46 from the surface of several cancer cell lines, and as a consequence render them more sensitive to antibody-dependent complement lysis. For example, Ad35K++ promotes killing of several lymphoma cell lines by rituximab, a humanized IgG1 targeting CD20 [25]. Moreover, *in vivo* administration of Ad35K++ to non-human primates, that ubiquitously express CD46, appears to be safe and well tolerated [26]. Therefore, using recombinant proteins targeting CD46 may be of potential use in clinical trials. Considering the key role of the CD46 pathway in controlling human T cell activation, we investigated herein the effect of Ad35K++ on T cell responses. We also assessed the effects of Ad35K- that has a point mutation (Arg279) that affects binding to CD46 [25]. Our data show that Ad35K++ strongly affected CD46 expression at the surface of primary T cells, and importantly that it was able to significantly impair T cell activation. T cells co-activated in presence of Ad35K++ had normal induction of CD69, an early marker of T cell activation but failed to express high levels of CD25, which was correlated with decreased proliferation and reduced cytokine production. In contrast, Ad35K- surprisingly led to enhanced T cell activation, with increased proliferation and cytokine production. These data emphasize the potency of recombinant adenoviral proteins in modulating the CD46 pathway, and provide the rationale to further investigate their effects on the immune response in order to maximize their therapeutic potential.

Methods

Cell purification and activation: PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology), from venous blood from healthy donors obtained after informed consent. Naïve CD4⁺ T cells were negatively isolated using magnetic beads (Stem Cells, purification > 95%), and cultured in RPMI 1640 with 10% fetal calf serum at 0.5×10^6 per well in 48-well plate pre-coated with α -CD3 (OKT3, 5 μ g/ml) or α -CD3/ α -CD46 (OKT3, 5 μ g/ml, MC120.6, 10 μ g/ml) (MC120.6 was kindly provided by Dr. Chantal Rabourdin-Combe, France, and recognized the SCR1 domain of CD46). Activated T cells also received rhIL-2 (Life Technologies - 10U/ml) to induce a Tr1-like phenotype [9]. Ad35K++ and Ad35K- were added to the wells at the start of the culture at the concentrations indicated. In some experiments, GM6001, a broad MMP inhibitor, was also added to the culture (Sigma, 10 μ M) [14].

Ad35K++ and Ad35K-: Recombinant, synthetic proteins derived from the fiber knob adenovirus Ad35 serotype. Both Ad35K++ and Ad35K- were selected using a display library as previously described [27, 28], with the Ad35K++ version having greatly increased affinity for the CD46 target. The coding sequences were cloned into a prokaryotic expression vector and gene expression induced by the addition of IPTG to the *E. coli* culture medium. Cells were lysed and the proteins purified using a combination of nickel affinity chromatography and ion exchange chromatography. The final proteins had high purity by SDS-PAGE and did not contain appreciable amounts of endotoxin that could affect cellular readouts.

Cytokine detection: Cell culture supernatants from the 48-well plates (as described in the cell activation section) were collected after five days of stimulation, and both IL-10 and

IFN γ secretion was determined by ELISA specific for human IL-10 (BD Pharmingen, San Diego, CA) and IFN γ (Endogen, Rockford, IL).

Flow cytometry. The expression level of CD46, CD69 and CD25 was assessed by flow cytometry, by incubating the cells with the antibodies at 4°C for 20 min in FACS buffer (PBS containing 1% fetal calf serum). We used different antibodies against CD46 as described in the results section: anti-CD46-FITC (clone MEM-258 recognizing the SCR4 domain - Biolegend), anti-CD46-PE (clone 344519, R&D) or the MCI20.6 clone recognizing the SCR1 domain followed by anti-IgG1-FITC antibodies. For activation experiments, we used the following antibodies: anti-CD46-PE, anti-CD69-FITC (Biolegend) and anti-CD25-APC (Biolegend). Samples were run with a FACSCalibur and data analyzed using FlowJo. Relative expression to staining with the control was calculated by calculating the DMFI (MFI obtained with antibody - MFI obtained with isotype control). Staining for intracellular expression of CD46 was performed in FACS buffer containing 0.1 % saponin (Sigma) for 30 min at room temperature, after fixation of the cells. Proliferation was determined by pre-labeling purified T cells with eFluor 670 cell proliferation stain (eBioscience) before activation following the manufacturer's instructions, and assessing remaining fluorescence after 4 days.

Statistics. The groups were analyzed using Graphpad Prism software. Flow cytometry data were analyzed using the Wilcoxon test, when assessing paired samples. ELISA data are the average of duplicate wells, and the average obtained for the different donors were analyzed using the Wilcoxon test. All p-values are two-tailed and with a 95% confidence interval.

Results

Effect of Ad35K++ and Ad35K- on CD46 expression on Jurkat cells

We first examined the effect of incubating Jurkat cells, a T leukemia cell line, with Ad35K++ and Ad35K- on CD46 surface expression. Jurkat cells were cultured in the presence or absence of a fixed concentration of both proteins (20 µg/ml) for various periods of times, and CD46 expression was then assessed by flow cytometry using an anti-CD46-FITC antibody. Addition of Ad35K++ for 1hr led to a total lack of detection of CD46, and the lack of CD46 expression was still observed after 48hrs of culture (Fig 1A). A shorter kinetic experiment showed that a total lack of detection of CD46 was also observed after a 5 min incubation period (not shown). Incubation with Ad35K- led to a slight decrease in the level of CD46 detected after 24 and 48 hrs, suggesting a residual binding to CD46. We next incubated Jurkat cells with various concentrations of Ad35K++ ranging from 1 pg/ml to 20 µg/ml, for 3 hrs. A dose dependent decrease in CD46 detection was observed. We observed a total effect with 10 ng/ml while there was no more effect on CD46 expression at 1 pg/ml, with a partial effect observed at 100 pg/ml (Fig 1B). These data suggested that Ad35K++ binding to CD46 could either mask the epitope in CD46 that is recognized by the antibody used for detection, and/or induce its internalization or cleavage.

In order to determine whether Ad35K++ binding to CD46 was masking CD46 antibody binding sites, we performed some competition experiments using different anti-CD46 antibodies recognizing distinct epitopes. We assessed the anti-CD46-FITC Ab used above that recognizes the SCR4 domain; an anti-CD46-PE Ab whose binding site has not been identified but that did not compete with the FITC Ab (Fig 2A); and the MCI20.6 monoclonal antibody that recognizes the SCR1 domain. Jurkat cells were first incubated with Ad35K++ for 20 min on ice, washed and then stained with these different anti-CD46

antibodies. Again, the use of the anti-CD46-FITC Ab led to a total lack of detection of CD46, suggesting that binding of Ad35K++ to CD46 on Jurkat cells was masking the epitope of CD46 recognized by this clone (Fig 2B). The use of the anti-CD46-PE antibody only led to a partial decrease, suggesting a partial blockage. Importantly, there was no competition between the MCI20.6 clone and Ad35K++, indicating that MCI20.6 could be used to activate cells in the presence of Ad35K++.

Ad35K++ and Ad35K- affect the phenotype of primary human T cells

We next determined the effect of Ad35K++ and Ad35K- on expression of CD46 on primary T cells. Purified naïve CD4+ T cells were cultured with Ad35K++ or Ad35K-(500 ng/ml), and expression of CD46 assessed after 24hrs using the MCI20.6 antibody that does not compete with Ad35K++. As binding of Ad35K++ to CD46 in several lymphoma cell lines has been shown to induce CD46 internalization [25], we also assessed intracellular CD46 expression after cell permeabilization. Incubation of T cells with Ad35K++ for 24hrs resulted in a reduced surface expression of CD46 on these cells (Fig. 3A, 3B) while Ad35K- had a lesser effect. Incubation with Ad35K++ had no significant effect on intracellular CD46 expression. We also assessed whether downregulation of CD46 expression by Ad35K++ could be affected by lowering the temperature to 4°C. T cells were incubated with Ad35K++ or Ad35K- (500 ng/ml) at 4°C or 37°C, and CD46 expression assessed by flow cytometry. Downregulation of CD46 expression was only observed at 37°C (Fig. 3C). These data suggest that internalization and/or MMP-dependent shedding of CD46 was blocked [14]. We next investigated whether MMP inhibition could modulate the effect of Ad35K++ on CD46 expression in T cells. Naïve CD4+ T cells were cultured in presence or absence of Ad35K++ (500 ng/ml) and/or GM6001, a broad MMP inhibitor, and CD46 expression was monitored

by flow cytometry using the MCI20.6 antibody (Fig. 3D). Inhibition of MMP increased the levels of surface CD46 in Ad35K++-treated T cells. These data suggest that ligation of Ad35K++ to CD46 on primary T cells led to, at least, a partial shedding of CD46.

We next investigated whether activation of primary T cells was affected in the presence of Ad35K++ or Ad35K-. Purified human naïve CD4+ T cells were left unstimulated or were activated with pre-immobilized anti-CD3 or anti-CD3/anti-CD46 (using the MCI20.6 antibody that does not compete with Ad35K++), as previously described [9, 17], in the presence or absence of Ad35K++ or Ad35K- (500 ng/ml). CD46 surface expression, as well as expression of the activation markers CD69 and CD25, was examined by flow cytometry after 2 and 5 days (Fig 4). As expected, CD3/CD46 costimulation of control cells induced a strong downregulation of CD46 surface expression, and this was correlated with an increased expression of CD69 and CD25, at both day 2 and day 5 [14]. In the presence of Ad35K++, a marked decrease in CD46 surface expression was observed in all conditions of activation, an effect that was still observed after 5 days of culture. The presence of Ad35K++ did not affect the induction of CD69 expression in T cells activated with either anti-CD3 or anti-CD3/CD46. In contrast, the presence of Ad35K++ significantly impaired CD25 induction in CD3/CD46-costimulated T cells. As previously observed, a slight decrease in surface CD46 expression was noted with Ad35K-. Surprisingly, Ad35K- significantly enhanced CD69 induction on CD3-activated T cells compared to control cells, and a significant effect, albeit more moderate, was also observed for CD25 expression. These data suggest that while Ad35K++ blocks late CD46-mediated T cell costimulation, the residual binding of Ad35K- to CD46 is sufficient to costimulate T cells activated by CD3.

Ad35K++ and Ad35K- modulate primary human T cell responses

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In order to determine whether these changes in phenotype would affect T cell functions, we next examined T cell responses. Naive T cells were stained with eFluoro670 before activation with immobilized anti-CD3 or anti-CD3/anti-CD46, in the presence of Ad35K++ or Ad35K-, and cell proliferation was determined after 4 days (Fig 5A). As expected, control cells proliferated upon CD3 activation and the proliferation was further increased upon CD3/CD46 costimulation [8]. Cells incubated in presence of Ad35K++ proliferated in response to CD3 ligation but failed to show increased proliferation upon CD3/CD46 costimulation. In contrast, cells cultured in the presence of Ad35K- showed increased proliferation in response to CD3 ligation compared to control cells, but no co-stimulation was observed following CD3/CD46 co-ligation.

Next, we determined cytokine production of cells activated in the presence of Ad35K++ and Ad35K-. As CD46 costimulation controls production of IFN γ and IL-10 [15], we assessed the levels of these 2 cytokines in the culture supernatants of primary T cells activated for 5 days in the presence of Ad35K++ or Ad35K- (Fig 5B). Cells costimulated in the presence of Ad35K++ secreted reduced amounts of cytokines compared to control cells. In contrast, T cells costimulated in the presence of Ad35K- secreted increased levels of IL-10 when compared with control cells or cells incubated with Ad35K++.

Discussion

In the past decade, the complement regulator CD46 has been of increasing interest to immunologists as several groups reported that its ligation on human T cells could profoundly affect T cell functions. While the first studies highlighted the role of CD46 ligation on T cell activation and proliferation [8, 11], further reports have demonstrated its key role in the control of inflammation by regulating not only T cell activation but also its ability to differentiate T cells towards an anti-inflammatory phenotype [9, 10, 12]. Strikingly, this pathway is dysregulated in a number of pathologies (MS, RA and asthma), which underlines

the key role of CD46 in the control of inflammation [15, 17-20]. Moreover, the role of CD46 as a cellular receptor for multiple pathogens suggests that this is a powerful pathway that can be exploited to the pathogens' advantage [29]. Therefore, specifically targeting CD46 may have potential use in several clinical settings.

Ad35K++ is a small recombinant protein derived from the fiber knob of the adenovirus serotype 35 (AD35) that uses CD46 as its receptor. It has been previously shown that Ad35K++ could increase the complement-dependent lysis of tumor cells lines [25], highlighting again the attractiveness of this drug to improve antibody-based cancer treatments. This suggestion is further supported by the fact that injection of Ad35K++ to monkeys, the only mammals that express CD46 in a pattern resembling humans, appears to be safe and well tolerated, and indeed *in vivo* administration of Ad35K++ could increase B cell depletion by rituximab [26].

Herein, we have focused on the effects of Ad35K++ and of a mutant Ad35K- on human T cell responses. We have examined CD46 expression, and response of T cells costimulated by CD46 in the presence of both recombinant proteins. Our data show that Ad35K++ binds to CD46 at the surface of primary T cells, and this interaction appears to be very stable as decreased CD46 expression was still detected after 5 days of culture. Of note, intravenous administration of Ad35K++ to macaques results in a decreased expression of CD46 on PBMCs that was still observed after 2 weeks post injection [26]. These data may be explained by the picomolar affinity of Ad35K++ for CD46. Previous studies have shown internalization of CD46 after binding to Ad35K++ in lymphoma cell lines [25]. Our results from experiments to examine intracellular staining of CD46 in primary T cells do not completely support internalization of CD46 in primary T cells, although downregulation of CD46 was blocked at 4°C, and we cannot exclude CD46 internalization and rapid

degradation. Our data also suggest that binding of Ad35K++ to CD46 leads to a partial MMP-dependent cleavage as partially blocked by addition of a broad MMP inhibitor.

The structure of the CD46 ectodomain resembles the shape of a 'hockey stick' [30], which likely explains the data obtained with the different clones of anti-CD46 antibodies used in the competition experiments with Ad35K++. Binding of Ad35K++ to the SCR1/2 domains of CD46 may also cause changes in CD46 conformation, as it totally competes with the anti-CD46-FITC Ab that recognizes the SCR4 domain [31]. Importantly, binding of Ad35K++ to CD46 does not affect recognition by the MCI20.6 Ab that also recognizes the SCR1 domain, allowing us to use this antibody to determine the effect of Ad35K recombinant proteins on CD46 costimulation. Our data show that early T cell activation appears to be normal in Ad35K++ treated cells, as we could observe a strong costimulation effect on CD69 induction between CD3 and CD3/CD46 costimulated T cells. However, there was no further increase in CD25 in CD46-costimulated T cells in presence of Ad35K++, especially after longer activation periods, and CD46-mediated costimulation was strongly impaired, as evidenced by reduced proliferation and cytokine production. Altogether, these data indicate that, although early T cell co-activation may remain unchanged, Ad35K++ blocks late T cell costimulation. Interestingly, recombinant Ad35 fiber knob proteins could inhibit CD3/CD28-mediated T cell activation, albeit inhibition could only be observed when the Ad35 knob was immobilized [32, 33], suggesting that multimerisation was required to suppress activation, possibly by hindering the spatial organization of the immune synapse. Therefore, Ad35K++ is a potent protein that strongly impairs the CD46-mediated pathway, at least *in vitro*.

Our data show a surprising effect of Ad35K- on T cell functions. This result was unexpected as Ad35K- was designed as a negative control, having a point mutation supposed to abrogate CD46 binding. However, incubation of primary T cells with Ad35K- led to a

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significant decrease in CD46 expression (see Fig 4B), indicating that there is a residual binding of Ad35K- to CD46. Indeed, binding of Ad35K- to several human cells lines were also observed (data not shown). Moreover, addition of Ad35K- was able to modulate T cell responses, providing a costimulatory signal to CD3-activated T cells, and leading to enhanced secretion of IL-10, suggesting its ability to promote Tr1 differentiation. Hence, in contrast to Ad35K++, Ad35K- acts as a CD46 agonist, which may be useful to boost the CD46 pathway *in vivo*.

In conclusion, both recombinant proteins differently modulate primary T cell responses, at least *in vitro*. Therefore, caution should be exerted in targeting this pathway, as ligation of CD46 can either promote or inhibit activation, likely depending on conformational changes triggered by ligand binding and subsequent signaling cascades. Nevertheless, our data emphasize the potency of recombinant proteins to modulate the CD46-mediated pathway, which is key to control immune homeostasis. The analysis of the effects of these two recombinant proteins on other immune cell types for which ligation of CD46 has been shown to modulate their response, such as dendritic cells [34] and B cells [35] will further support the development of these potential drugs into clinical trials.

Acknowledgements

We thank Dongqing Ma who performed some preliminary experiments during her Master. JH has done the experiments, AB and DC have provided the recombinant proteins, have performed experiments (data not shown) and discussed data; AA has written the manuscript. We thank Fiona Rossi and Shonna Johnston for their help with flow cytometry. We are grateful to Ian Dransfield for the critical reading of the manuscript. AA is an MRC academic fellow and detached member of CNRS, France. JH is an MRC-PhD student. AL and DC were supported by NIH grant 2R44CA162582-02.

Competing Interests statement

The authors declare no competing interests.

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Figure Legends

Figure 1. Ad35K++ affects the detection of CD46 expression on Jurkat cells. (A) Jurkat cells were incubated with Ad35K++ or Ad35K- (20 µg/ml) for various lengths of time, as indicated. CD46 surface expression was then monitored by flow cytometry using an anti-CD46-FITC antibody. (B) Jurkat cells were incubated for 3h with various concentrations of Ad35K++ as indicated and CD46 surface expression was monitored by flow cytometry using the anti-CD46-FITC antibody. Dotted lines represent isotype controls.

Figure 2. Competition experiments between Ad35K++ and anti-CD46 antibodies. (A) Jurkat cells were stained with anti-CD46-FITC or anti-CD46-PE, or with both antibodies added in the different orders, as indicated, and CD46 expression was assessed by flow cytometry. These two clones do not compete with each other. (B) Jurkat cells were first incubated with Ad35K++ (500 ng/ml) for 20 min, washed and then stained with the anti-CD46-FITC, anti-CD46-PE, or indirectly labeled with the MCI20.6 clone followed by anti-mIgG1-FITC. CD46 expression was assessed by flow cytometry. Dotted lines represent isotype controls.

Figure 3. Ad35K++ affects expression of surface CD46 on primary naïve CD4+ T cells. (A) Naïve T cells were cultured with Ad35K++ or Ad35K- (500 ng/ml) for 24h, and CD46 expression was determined at the cell surface or after intracellular staining using the

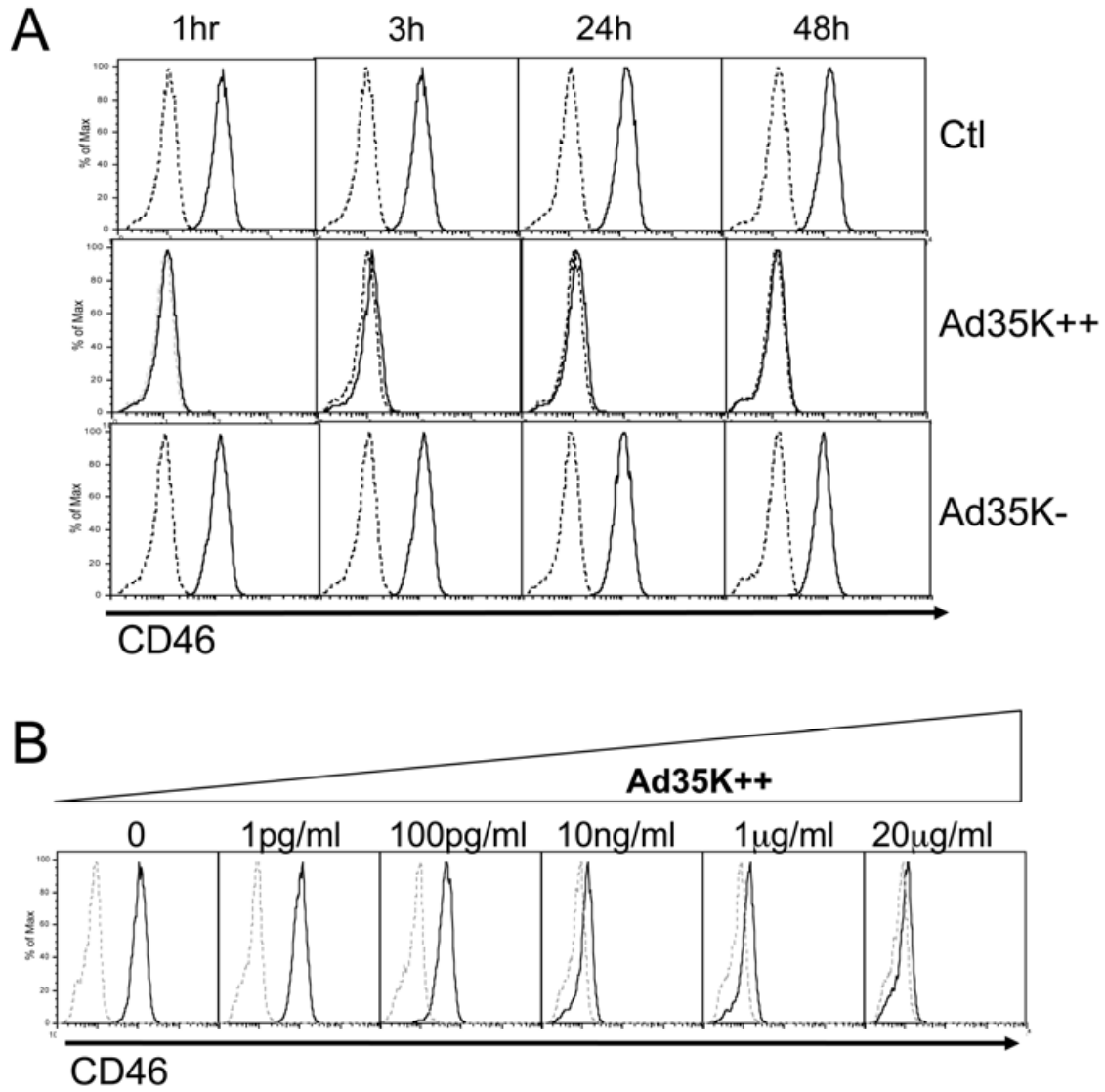
MCI20.6 clone that does not compete with Ad35K++. Dotted lines represent isotype controls. **(B)** Represents the data obtained with 3 different donors. **(C)** Naive T cells were cultured with Ad35K++ or Ad35K- (500 ng/ml) for 24h either at 4°C or 37°C, and CD46 expression was then determined using the MCI20.6 MAb. Representative of 2 experiments. **(D)** Naive T cells were cultured in the presence of Ad35K++ (500 ng/ml) and with or without addition of GM6001, a broad MMP inhibitor. Forty-eight hours later, CD46 surface expression was then analyzed by flow cytometry using the MCI20.6 antibody.

Figure 4. Ad35K++ impairs induction of CD25 while Ad35K- promotes costimulation.

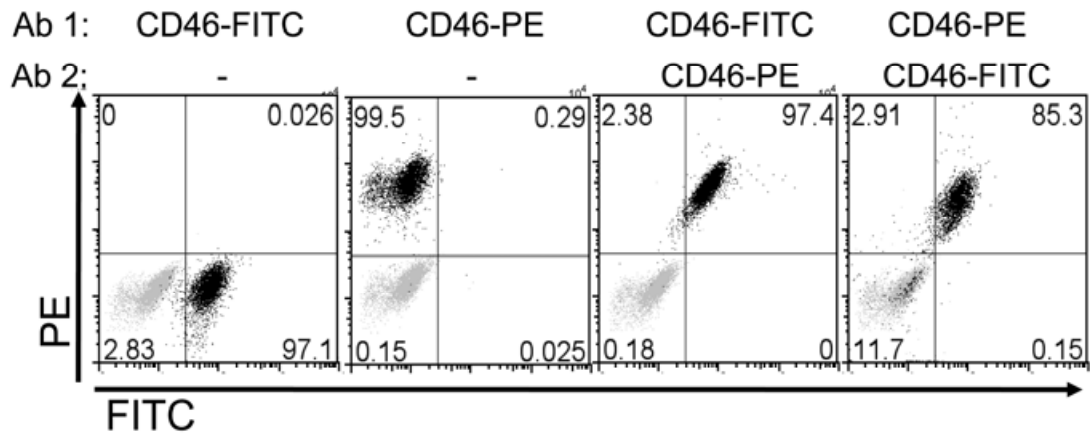
Naïve CD4+ T cells were left unstimulated (US) or were activated by immobilized anti-CD3 or anti-CD3/anti-CD46 for 2 or 5 days in the presence or absence of Ad35K++ or Ad35K- (500 ng/ml). Expression of CD46 (using the anti-CD46-PE clone), CD69 and CD25 were assessed. Dotted lines represent isotype controls. **(A)** shows the raw data obtained for one donor at day 2, and **(B)** the data obtained for the different donors analyzed at day 2 and day 5 (n=6).

Figure 5. Ad35K++ inhibits T cell costimulation while Ad35K- increases activation.

(A) Purified naïve CD4+ T cells were pre-labeled with eFluor 670 before activation by immobilized anti-CD3 or anti-CD3/anti-CD46 in the presence or absence of Ad35K++ or Ad35K-. Four days later, proliferation was monitored by flow cytometry. **(B)** The production of IL-10 and IFN γ in the culture supernatants was assessed by ELISA (n=6).



A



B

